4-9-01 JC04 Rec'd PCT/PTO 06 APR 2001

FORM PTO-1390 (REV 11-2000) | U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE | ATTORNEY'S DOCKET NUMBER: Goudreau

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371

U.S APPLICATION NO (If known, see 37 CFR 1.5): 09/807047

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA99/00933	November 6, 1999	November 7, 1998

TITLE OF INVENTION

OLIGONUCLEOTIDE PRIMERS THAT DESTABILIZE NON-SPECIFIC DUPLEX FORMATION AND USES THEREOF

APPLICANT(S) FOR DO/EO/US

Jerry PELLETIER and Manjula DAX

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application under PCT Article 19 (35 U.S.C 371(c)(2))
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5))

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3 31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information: Copy of response to Written Opinion originally submitted December 5, 2000; return receipt postcard.

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

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I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

JVenegas

Tamara Venegas

pa-579904

U.S. APPLICATION NO (if known, see 37 CFR 1.5) 09/807047	INTERNATIONAL APPLICATION NO. *	ATTORNEY'S DOCKET NUMBER *

21. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....$1,000.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....$860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provision of PCT Article 33(1)-(4) $690.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00

	CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$1000	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$0	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	36 - 20 =	16	x $18.00	$288	
Independent claims	12 - 3 =	9	x $80.00	$720	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$0	
			TOTAL OF ABOVE CALCULATIONS =	$2008	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.				$1004	
			SUBTOTAL =	$1004	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$0	
			TOTAL NATIONAL FEE =	$1004	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00 per property**			+	$0	
			TOTAL FEES ENCLOSED =	$1004	
			Amount to be refunded:	**$0**	
			charged:	**$1004**	

a. ☐ A check in the amount of $ to cover the above fees is enclosed.

b. ☒ Please charge my **Deposit Account No. 03-1952** in the amount of **$1004** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment to **Deposit Account No. 03-1952**. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Gladys H. Monroy
Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, California 94304-1018

Gladys Monroy
SIGNATURE

Gladys H. Monroy
Registration No. 32,430

PATENT
Docket No. GOUDREAU
Client Reference CG/11168.115

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

Express Mail Label No EL 569 177 315 US Date of Deposit. April 6, 2001

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TVenegas
Tamara Venegas

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Jerry PELLETIER and Manjula DAS

Serial No.: To Be Assigned

Filing Date: Herewith

For: OLIGONUCLEOTIDE PRIMERS THAT DESTABILIZE NON-SPECIFIC DUPLEX FORMATION AND USES THEREOF

Examiner: To Be Assigned

Group Art Unit: To Be Assigned

PRELIMINARY AMENDMENT

Box PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to examination of the above-captioned application, please enter the following amendments and remarks.

AMENDMENTS

In the Claims:

4. (Amended) The method of claim 1, wherein said oligonuclotide is a homopolymer comprising at least one nucleotide modification.

13. (Amended) The method according to claim 5, wherein an enzyme capable of RNA-dependent DNA polymeriztion is used for said first strand synthesis.

19. (Amended) The method of claim 16, wherein said oligonucleotide is a homopolymer.

REMARKS

Attached hereto is a marked-up version of the changes made ot the specification and claims by the current amendment. The attached page is captioned "**Version with markings to show changes made**".

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. **GOUDREAU**. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: April 6, 2001 By: Gladys Monroy

Gladys H. Monroy
Registration No. 32,430

Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, California 94304-1018
Telephone: (650) 813-5711
Facsimile: (650) 494-0792

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

4. The method of [one of] claim[s] 1[-3], wherein said oligonucleotide is a homopolymer comprising at least one nucleotide modification.

13. The method according to [one of] claim[s] 5 [to 12], wherein an enzyme capable of RNA-dependent DNA polymerization is used for said first strand synthesis.

19. The method of claim[s] 16, [17 or 18,] wherein said oligonucleotide is a homopolymer.

PATENT
Docket No. 514012000100
Client Reference 760/11168.163

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

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Tamara Alcaraz

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Jerry PELLETIER and Manjula DAS

Application No.: 09/807,047

Filing Date: April 6, 2001

For: OLIGONUCLEOTIDE PRIMERS THAT DESTABILIZE NON-SPECIFIC DUPLEX FORMATION AND USES THEREOF

Examiner: To Be Assigned

Group Art Unit: To Be Assigned

SUPPLEMENTAL PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to examination of the above-captioned application, please enter the following amendments and remarks.

pa-681352

AMENDMENTS

In the Claims:

Please replace the complete set of claims by the new set submitted herewith.

1. A method for destabilizing non-specific duplex formation between a homopolymeric sequence of an oligonucleotide and a non-homopolymeric target nucleic acid, comprising a modification of said homopolymeric sequence of said oligonuclotide, wherein said modification decreases or abrogates hydrogen bonding between said modified homopolymeric sequence of said oligonucleotide and a non-homopolymeric target sequence.

2. The method of claim 1, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

3. The method of claim 2, wherein said universal base is 3-nitropyrrole.

4. The method of claim 1, wherein said oligonucleotide is a homopolymer comprising at least one nucleotide modification.

5. A method for increasing the proportion of full length cDNA clones in a library, comprising a use of a modified oligo d(T) homopolymer during first strand synthesis, wherein said modified oligo d(T) homopolymer comprises a modification which decreases or abrogates hydrogen bonding between said modified oligo d(T) homopolymer and a non-homopolymeric target sequence, thereby increasing the proportion of full length cDNA clones.

6. The method of claim 5, wherein said modification is at least one universal base incorporated into said oligo d(T) homopolymer.

7. The method of claim 6, wherein said universal base is 3-nitropyrrole.

8. The method of claim 5, wherein said modification is at least one chemically modified nucleoside incorporated into said oligo d(T) homopolymer.

9. The method of claim 5, wherein said modification is at least one base analog incorporated into said oligo d(T) homopolymer.

10. The method of claim 9, wherein said base analog is inosine.

11. The method of claim 5, wherein said modification is at least one mismatch incorporated into said oligo d(T) homopolymer.

12. The method of claim 5, wherein said modification is a phosphate or ribose modification incorporated into said oligo d(T) homopolymer.

13. The method according to claim 5, wherein an enzyme capable of RNA-dependent DNA polymerization is used for said first strand synthesis.

14. The method according to claim 13, wherein said enzyme is a reverse transcriptase selected from the group consisting of avian myoblastoid virus reverse transcriptase, murine moloney leukemia virus reverse transcriptase, and human immuno deficiency virus reverse transcriptase.

15. A kit for the synthesis of cDNA, said kit comprising a modified oligo d(T) homopolymeric primer, wherein said modified oligonucleotide includes a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

16. A method for reducing mispriming events during DNA synthesis comprising a use of a modified oligonucleotide to prime said DNA synthesis, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby reducing mispriming events, while maintaining a formation of a duplex with a homopolymeric target sequence.

17. The method of claim 16, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

18. The method of claim 17, wherein said universal base is 3-nitropyrrole.

19. The method of claim 16, wherein said oligonucleotide is a homopolymer.

20. A method for reducing mispriming during 5' RACE comprising a use of a modified oligonucleotide to prime said 5' RACE, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby reducing mispriming events while maintaining a formation of a duplex with a homopolymeric target sequence.

21. The method of claim 20, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

22. The method of claim 20, wherein said universal base is 3-nitropyrrole.

23. The method of claim 22, wherein said modification is at least one chemically modified nucleoside incorporated into said homopolymeric sequence.

24. The method of claim 20, wherein said modification is at least one base analog incorporated into said homopolymeric sequence.

25. The method of claim 24, wherein said base analog is inosine.

26. The method of claim 20, wherein said modification is at least one mismatch incorporated into said homopolymeric sequence.

27. The method of claim 20, wherein said modification is a phosphate or ribose modification incorporated into said homopolymeric sequence.

28. A kit for 5' RACE comprising a modified oligonucleoticle primer, comprising a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

29. A method for reducing mispriming during 3' RACE comprising a priming of said 3' RACE with a modified oligonucleotide, wherein said modified oligonucleoticle comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby reducing mispriming events while maintaining a formation of a duplex with a homopolymeric target sequence.

30. The method of claim 29, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

31. A method for generating *bona fide* genetic markers comprising a use of a modified oligonucleoticle to prime from homopolymeric stretch, wherein said modified oligonucleoticle comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

32. The method of claim 31, wherein said modified oligonucleotide primes from an internal A-rich region in an Alu repeat.

33. A method for stabilizing duplex formation between an oligonucleotide comprising a homopolymeric sequence and a target homopolymeric sequence comprising a modification of said homopolymeric sequence of said oligonucleotide, wherein said modification decreases or abrogates hydrogen bonding between same and non-homopolymeric target sequence, thereby stabilizing duplex formation between said oligonucleotide and said target sequence.

34. A method for reducing mispriming during sequencing comprising a use of a modified oligonucleotide to prime DNA synthesis, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

35. A method to improve the discrimination between a binding of an oligonucleotide homopolymeric sequence to its targeted homopolymeric sequence versus a non-homopolymeric sequence comprising an insertion into a homopolymeric sequence of said oligonucleotide of at least one modification which decreases or abrogates hydrogen bonding between same and said non-homopolymeric sequence.

36. A method for increasing the proportion of full length cDNA clones in a library, comprising a use of a modified oligonucleotide during second strand synthesis from a 3' end-tailed first strand product, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby increasing the proportion of full length cDNA clones.

REMARKS

The new set of claims, which is now presented, is based on the original set thereof, amended in view of the Written Opinion and to better define the subject-matter of the present invention.

More specifically, claim 14 has been canceled since it was a duplicate of claim 13. Accordingly, claims 15-37 have been renumbered so as to become claims 14-36. Such language is amply supported by the disclosure, but specific support can be found, for example, at page 6, from lines 20 to 26. These amendments do not constitute new matter.

In view of the amendments to the claims and the arguments submitted above, it is respectfully submitted that the claims, which now more specifically relate to the destabilization between a modified homopolymeric region of an oligo and a non-homopolymeric sequence of a target nucleic acid, are novel and inventive.

In the unlikely event that the Patent Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. **514012000100**. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: April 8, 2002

By: _____

Gladys H. Monroy
Registration No. 32,430
Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, California 94304-1018
Telephone: (650) 813-5711
Facsimile: (650) 494-0792

WHAT IS CLAIMED IS:

1. A method for destabilizing non-specific duplex formation between a homopolymeric sequence of an oligonucleotide and a non-homopolymeric target nucleic acid, comprising a modification of said homopolymeric sequence of said oligonucleotide, wherein said modification decreases or abrogates hydrogen bonding between said modified homopolymeric sequence of said oligonucleotide and a non-homopolymeric target sequence.

2. The method of claim 1, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

3. The method of claim 2, wherein said universal base is 3-nitropyrrole

4 The method of one of claims 1-3, wherein said oligonucleotide is a homopolymer comprising at least one nucleotide modification.

5. A method for increasing the proportion of full length cDNA clones in a library, comprising a use of a modified oligo d(T) homopolymer during first strand synthesis, wherein said modified oligo d(T) homopolymer comprises a modification which decreases or abrogates hydrogen bonding between said modified oligo d(T) homopolymer and a non-homopolymeric target sequence, thereby increasing the proportion of full length cDNA clones.

6. The method of claim 5, wherein said modification is at least one universal base incorporated into said oligo d(T) homopolymer.

7. The method of claim 6, wherein said universal base is 3-nitropyrrole.

8. The method of claim 5, wherein said modification is at least one chemically modified nucleoside incorporated into said oligo d(T) homopolymer.

9. The method of claim 5, wherein said modification is at least one base analog incorporated into said oligo d(T) homopolymer.

10. The method of claim 9, wherein said base analog is inosine.

11. The method of claim 5, wherein said modification is at least one mismatch incorporated into said oligo d(T) homopolymer.

12. The method of claim 5, wherein said modification is a phosphate or ribose modification incorporated into said oligo d(T) homopolymer.

13. The method according to one of claims 5 to 12, wherein an enzyme capable of RNA-dependent DNA polymerization is used for said first strand synthesis.

14. The method according to claim 13, wherein said enzyme is a reverse transcriptase selected from the group consisting of avian myoblastoid virus reverse transcriptase, murine moloney leukemia virus reverse transcriptase, and human immuno deficiency virus reverse transcriptase.

15. A kit for the synthesis of cDNA, said kit comprising a modified oligo d(T) homopolymeric primer, wherein said modified

oligonucleotide includes a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

16. A method for reducing mispriming events during DNA synthesis comprising a use of a modified oligonucleotide to prime said DNA synthesis, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby reducing mispriming events, while maintaining a formation of a duplex with a homopolymeric target sequence.

17. The method of claim 16, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

18. The method of claim 17, wherein said universal base is 3-nitropyrrole.

19. The method of claims 16, 17 or 18, wherein said oligonucleotide is a homopolymer.

20. A method for reducing mispriming during 5' RACE comprising a use of a modified oligonucleotide to prime said 5' RACE, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby reducing mispriming events while maintaining a formation of a duplex with a homopolymeric target sequence.

21. The method of claim 20, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

22. The method of claim 20, wherein said universal base is 3-nitropyrrole.

23. The method of claim 22, wherein said modification is at least one chemically modified nucleoside incorporated into said homopolymeric sequence.

24. The method of claim 20, wherein said modification is at least one base analog incorporated into said homopolymeric sequence.

25. The method of claim 24, wherein said base analog is inosine.

26. The method of claim 20, wherein said modification is at least one mismatch incorporated into said homopolymeric sequence.

27. The method of claim 20, wherein said modification is a phosphate or ribose modification incorporated into said homopolymeric sequence.

28. A kit for 5' RACE comprising a modified oligonucleotide primer, comprising a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

29. A method for reducing mispriming during 3' RACE comprising a priming of said 3' RACE with a modified oligonucleotide, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby reducing mispriming events while maintaining a formation of a duplex with a homopolymeric target sequence.

30. The method of claim 29, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

31. A method for generating *bona fide* genetic markers comprising a use of a modified oligonucleotide to prime from a homopolymeric stretch, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

32. The method of claim 31, wherein said modified oligonucleotide primes from an internal A-rich region in an Alu repeat.

33. A method for stabilizing duplex formation between an oligonucleotide comprising a homopolymeric sequence and a target homopolymeric sequence comprising a modification of said homopolymeric sequence of said oligonucleotide, wherein said modification decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby stabilizing duplex formation between said oligonucleotide and said target sequence.

34. A method for reducing mispriming during sequencing comprising a use of a modified oligonucleotide to prime DNA synthesis, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

35. A method to improve the discrimination between a binding of an oligonucleotide homopolymeric sequence to its targeted homopolymeric sequence versus a non-homopolymeric sequence comprising an insertion into said homopolymeric sequence of said oligonucleotide of at

least one modification which decreases or abrogates hydrogen bonding between same and said non-homopolymeric sequence.

36. A method for increasing the proportion of full length cDNA clones in a library, comprising a use of a modified oligonucleotide during second strand synthesis from a 3' end-tailed first strand product, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby increasing the proportion of full length cDNA clones.

WHAT IS CLAIMED IS:

1. A method for destabilizing non-specific duplex formation between a homopolymeric sequence of an oligonucleotide and a non-homopolymeric target nucleic acid, comprising a modification of said homopolymeric sequence of said oligonucleotide, wherein said modification decreases or abrogates hydrogen bonding between said modified homopolymeric sequence of said oligonucleotide and a non-homopolymeric target sequence.

2. The method of claim 1, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

3. The method of claim 2, wherein said universal base is 3-nitropyrrole.

4 The method of one of claims 1-3, wherein said oligonucleotide is a homopolymer comprising at least one nucleotide modification.

5. A method for increasing the proportion of full length cDNA clones in a library, comprising a use of a modified oligo d(T) homopolymer during first strand synthesis, wherein said modified oligo d(T) homopolymer comprises a modification which decreases or abrogates hydrogen bonding between said modified oligo d(T) homopolymer and a non-homopolymeric target sequence, thereby increasing the proportion of full length cDNA clones.

6. The method of claim 5, wherein said modification is at least one universal base incorporated into said oligo d(T) homopolymer.

7. The method of claim 6, wherein said universal base is 3-nitropyrrole.

8. The method of claim 5, wherein said modification is at least one chemically modified nucleoside incorporated into said oligo d(T) homopolymer.

9. The method of claim 5, wherein said modification is at least one base analog incorporated into said oligo d(T) homopolymer.

10. The method of claim 9, wherein said base analog is inosine.

11 The method of claim 5, wherein said modification is at least one mismatch incorporated into said oligo d(T) homopolymer.

12. The method of claim 5, wherein said modification is a phosphate or ribose modification incorporated into said oligo d(T) homopolymer.

13. The method according to one of claims 5 to 12, wherein an enzyme capable of RNA-dependent DNA polymerization is used for said first strand synthesis.

14. The method according to claim 13, wherein said enzyme is a reverse transcriptase selected from the group consisting of avian myoblastoid virus reverse transcriptase, murine moloney leukemia virus reverse transcriptase, and human immuno deficiency virus reverse transcriptase.

15. A kit for the synthesis of cDNA, said kit comprising a modified oligo d(T) homopolymeric primer, wherein said modified

oligonucleotide includes a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

16. A method for reducing mispriming events during DNA synthesis comprising a use of a modified oligonucleotide to prime said DNA synthesis, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby reducing mispriming events, while maintaining a formation of a duplex with a homopolymeric target sequence.

17. The method of claim 16, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

18. The method of claim 17, wherein said universal base is 3-nitropyrrole.

19 The method of claims 16, 17 or 18, wherein said oligonucleotide is a homopolymer.

20. A method for reducing mispriming during 5' RACE comprising a use of a modified oligonucleotide to prime said 5' RACE, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby reducing mispriming events while maintaining a formation of a duplex with a homopolymeric target sequence.

21 The method of claim 20, wherein said modification is at least one universal base incorporated into said homopolymeric sequence

22. The method of claim 20, wherein said universal base is 3-nitropyrrole.

23. The method of claim 22, wherein said modification is at least one chemically modified nucleoside incorporated into said homopolymeric sequence.

24. The method of claim 20, wherein said modification is at least one base analog incorporated into said homopolymeric sequence.

25. The method of claim 24, wherein said base analog is inosine.

26. The method of claim 20, wherein said modification is at least one mismatch incorporated into said homopolymeric sequence.

27. The method of claim 20, wherein said modification is a phosphate or ribose modification incorporated into said homopolymeric sequence.

28. A kit for 5' RACE comprising a modified oligonucleotide primer, comprising a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

29. A method for reducing mispriming during 3' RACE comprising a priming of said 3' RACE with a modified oligonucleotide, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby reducing mispriming events while maintaining a formation of a duplex with a homopolymeric target sequence.

30. The method of claim 29, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

31. A method for generating *bona fide* genetic markers comprising a use of a modified oligonucleotide to prime from a homopolymeric stretch, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

32. The method of claim 31, wherein said modified oligonucleotide primes from an internal A-rich region in an Alu repeat.

33. A method for stabilizing duplex formation between an oligonucleotide comprising a homopolymeric sequence and a target homopolymeric sequence comprising a modification of said homopolymeric sequence of said oligonucleotide, wherein said modification decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby stabilizing duplex formation between said oligonucleotide and said target sequence.

34. A method for reducing mispriming during sequencing comprising a use of a modified oligonucleotide to prime DNA synthesis, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence.

35. A method to improve the discrimination between a binding of an oligonucleotide homopolymeric sequence to its targeted homopolymeric sequence versus a non-homopolymeric sequence comprising an insertion into said homopolymeric sequence of said oligonucleotide of at

least one modification which decreases or abrogates hydrogen bonding between same and said non-homopolymeric sequence.

36. A method for increasing the proportion of full length cDNA clones in a library, comprising a use of a modified oligonucleotide during second strand synthesis from a 3' end-tailed first strand product, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and a non-homopolymeric target sequence, thereby increasing the proportion of full length cDNA clones.

TITLE OF THE INVENTION

OLIGONUCLEOTIDE PRIMERS THAT DESTABILIZE NON-SPECIFIC DUPLEX FORMATION AND USES THEREOF.

FIELD OF THE INVENTION

The present invention relates to genetic engineering. More specifically, a method is presented for reducing mispriming during DNA synthesis. In particular, the present invention relates to primers containing modified nucleosides (e.g. universal base) which reduce mispriming during cDNA library construction, thereby increasing the proportion of cDNA clones having been primed from the *bona fide* 3' poly A tail. The present invention further relates to the use of the discriminating oligonucleotides of the present invention in other methods such as mRNA purification, PCR-based detection methods and sequencing.

BACKGROUND OF THE INVENTION

The isolation and rapid mapping of complementary DNAs (cDNAs) is central to characterizing the information that is .of significant biological relevance in the genome of an organism. A full length cDNA allows one to predict transcription initiation start sites, translation initation start sites, deduce certain protein characteristics based on primary amino acid sequence, predict transcription termination sites, and visually inspect the 5' and 3' untranslated regions for elements which may be involved in post-transcriptional regulation of gene expression. The analysis of several complete cDNAs of a given gene enables one to gather information on alternative splicing, alternative promoter usage, and alternative polyadenylation signals - all events known to be important in gene expression regulation. In addition, the comparison of genomic and cDNA sequence is essential to determine exon-intron structure and document the occurrence of RNA editing - a post-transcriptional regulatory mechanism on which there is little information.

The cloning of mRNA into cDNA for the purposes of functional studies is a complex, interrelated series of enzyme-catalyzed reactions involving the *in vitro* synthesis of a DNA copy of mRNA, its subsequent conversion to duplex cDNA, and insertion into an appropriate prokaryotic vector. The procedure may involve the following series of steps (outlined in Fig.1):

1) Isolation of high quality mRNA from the tissue or cell line of interest

2) Annealing of a DNA oligonucleotide, either a mixture of oligonucleotides of random sequence or an oligo d(T) primer, to the mRNA When full-length cDNAs are required, oligo d(T) is utilized, since this is expected to anneal to the 3' poly (A) tail of the mRNA

3) Reverse transcriptase is then utilized to prime from the DNA primer and copy the RNA template into cDNA.

4) Second strand synthesis is performed utilizing RNAse H, DNA polymerase I, and DNA ligase.

5) The ends of the cDNAs are polished, prepared for cloning, and the cDNAs are introduced into an appropriate cloning vector.

Although a number of different approaches can be used to generate cDNA libraries, they suffer from several major problems, often making the isolation of a complete cDNA an arduous task The cloning of incomplete cDNAs is widespread, resulting in only partial characterization of mRNA transcripts and significantly increasing the cost and amount of work required to obtain a full-length copy of the cDNA of interest. One major reason why many clones in current cDNA libraries are not full-length is due to mispriming of the oligo d(T) primer (de Fatima Bonaldo et al., 1996, *Genome Res* 6:791-806). Many eukaryotic mRNAs contain regions of A-rich stretches within their sequence. Thus oligo d(T) primers can anneal to these internal A-rich stretches When reverse transcriptase primes from these internal sites, sequence information from the 3' end of the mRNA is lost during the cDNA cloning process (Fig. 1). Although the genetic code of most organisms is composed of ~ 50%

guanosine + cytosine residues and 50% of adenosine + thymidine residues, there are well known examples of organisms whose genetic code deviates from this ratio. For example, the genome of the parasite responsible for malaria transmission, *Plasmodium falciparum*, has a genome of >80% adenosine + thymidine residues (Weber, J.L., 1987, *Gene* 52:103-109). This implies that cDNA libraries derived from this organism will contain many truncated, less-than-full-length clones, due to mispriming of the oligo d(T) primer during first strand synthesis. Mispriming is thus a serious hindrance to gene discovery and characterization in general, and more acutely for certain organisms.

These technical limitations imply that a set of products of variable length are often generated during first strand synthesis. Consequently, a number of truncated clones may be present in any given library. Given these cloning complications, interpretations about gene structure are sometimes misleading and cDNA cloning is often inefficient, costly, and time-consuming - often requiring the sampling of several different libraries.

The actual procedure for generating cDNA libraries has not extensively deviated from the original method of Gubler et al., 1983, *Gene* 25:263-269. Because of the frequent generation of products of variable length during first strand synthesis, a number of truncated clones will be present in libraries for any given gene. Priming from the poly (A) tract of mRNAs with oligo d(T) is necessary to obtain a copy of the entire 3' untranslated region. However, it is the experience of many laboratories screening cDNA libraries, that a significant proportion of clones do not have a *bona fide* 3' end, due to misannealing of the oligo d(T) primer to internal A-rich sites. Indeed, cDNAs with 3' truncations are estimated to occur at frequencies of 10-15% in some libraires (de Fatima Bonaldo et al., 1996, *supra*). Such clones are easily recognized by the absence of a *bona fide* polyadenylation signal sequence ~20 nucleotides upstream of the oligo (dA) tail of the cDNA. If enhanced discrimination could be achieved between annealing to the *bona fide* poly (A) tail versus internal A-rich

sequences by the Reverse Transcriptase primer, then the frequency of this "mispriming artifact" would be significantly reduced.

Nucleic acid hybridization, in which a DNA or RNA strand binds to its complement to form a duplex structure is a fundamental process in molecular biology. A critical aspect of this process is the specificity of molecular recognition of one strand by the other. Sequence differences as subtle as a single base change are sufficient to enable discrimination of short (e.g. - 14 mer) oligomers, and are frequently used to detect point mutations in genes (Conner et al., 1983, *Proc. Natl. Acad. Sci. USA.* 80:278-282.). Molecular discrimination of single point changes using oligonucleotides has been well documented and the underlying thermodynamics well characterized (Ikuta et al., 1987, *Nucl. Acids Res.* 15:797-811; Doktycz et al., 1995, *J. Biol. Chem.* 270:8439-8445; Southern et al., 1994, *Nucl. Acids Res.* 22:1368-1373; Saiki et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:6230-6234). However, in many cases, the stability difference between a perfectly matched complement (e.g. - between a poly (A) tail and oligo $d(T)_{15}$) and a complement mismatched at only one base (e.g. - between AAAAAAATAAAAAAA and oligo $d(T)_{15}$) can be quite small, corresponding to as little as 0.5°C difference in their duplex melting temperature (T_ms) (Fig. 2). The longer the oligomer of interest (e.g. an oligo $d(T)_{20}$ primer versus and oligo $d(T)_{15}$ primer) the smaller the effect of a single-base mismatch on overall duplex stability. This limitation in hybridization is the major reason why oligo d(T) primers often hybridize to internal A-rich sequences on mRNA templates during cDNA library construction, and consequently why a large number of clones in such libraries do not contain the *bona fide* 3' end.

Guo et al. (1997, *Nature Biotech.* 15:331-335) have recently shown that increased discrimination of single nucleotide mismatches by oligonucleotides can be achieved by introducing artifical mismatches into the probe oligonucleotide using the base analog 3-nitropyrrole. This base analog acts as a universal nucleoside that hydrogen bonds minimally with all four bases without steric disruption of the DNA duplex (Nichols et al., 1994, *Nature*

369:492-493). Since hydrogen bonding between bases of two complementary strands of DNA is the major thermodynamic force responsible for maintaining the integrity of a double stranded DNA duplex, base substitutions with analogs with lessened hydrogen bond capacity can function as universal nucleosides (Nichols et al., 1994, *supra*). A number of different nucleoside analogs have been developed which function in this fashion (Millican et al., 1984, *Nucl. Acids Res.* 12:7435-7453; Inone et al., 1985, *Nucl. Acids Res.* 13:7119-7128; Fukada et al., 1986, *Naturforsch. B.* 41:1571-1579; Seela et al., 1986, *Nucl. Acids Res.* 14:1825-1844; Eritja et al., 1986, *Nucl. Acids Res.* 14:8135-8153; Habener et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:1735-1739; Lin et al., 1989, *Nucl. Acids Res.* 17:10373-10383; Francois et al., 1990, *Tetrahedron Lett.* 31:6347-6350; Brown et al., 1991, *Carbohydrate Res.* 216:129-139.). Guo et al. (1997, *supra)* have shown that the introduction of universal analogues into heteropolymeric oligonucleotides during their synthesis, increases the thermal stability ($_{\Delta}T_m$) of hybrids formed between an oligonucleotide with the universal nucleoside and normal and single-nucleotide variant DNA targets by as much as 200%, as compared to hybrids formed between a wild-type oligonucleotide and normal or single-nucleotide variant DNA targets.

U.S. patent 5,438,131 of Bergstrom et al. teaches oligonucleotides of at least 10 nucleosides, composed of at least two different bases, and containing at least one universal nucleoside and the use thereof to reduce the element of risk and enhance success in screening DNA libraries. The universal base is defined in U.S. 5,438,131 as being a modified nucleic acid base that can base-pair with its ally, one of the common bases A, T, C and G (as well as U). The aim of the universal base is to reduce degeneracy while still preserving the uniqueness of the probe. A variety of compounds have been investigated as universal bases and a number of them are described in U.S. 5,438,131. In a preferred embodiment, U.S. 5,438,131 relates to oligonucleotides containing universal nucleosides at degenerate positions, such that the oligomer allows bonding to unknown bases, enabling the formation of

duplexes with ambiguous or unknown nucleic acid sequences. In a particularly preferred embodiment, U.S. 5,438,131 relates to 3-nitropyrrole nucleoside as the universal nucleoside. U.S. 5,438,131 thus relates to the use of universal nucleosides in order to stabilize duplex formation between heteropolymers of oligonucleotides and a target nucleic acid.

In view of the technical limitations of current methods of cDNA synthesis, there remains a need to destabilize artefactual duplex formation to increase the discrimination between specific and non-specific duplexes. There also remains a need to provide the means to reduce mismatches in general, and more particularly to reduce mispriming during DNA synthesis, cDNA library construction, and PCR applications. The present invention seeks to meet these and other needs.

The present description refers to a number of documents, the content of which is herein incorporated by reference, in their entirety.

SUMMARY OF THE INVENTION

The invention concerns the identification of primer modifications that can destabilize artifactual duplex formation and decrease the number of mismatches between the primer and its target sequence.

In one embodiment, the invention further concerns the identification of primer modifications that improve the discrimination between the binding thereof to a homopolymeric target sequence (the *bona fide* target sequence) as compared to a non-homopolymeric target sequence. The invention therefore provides oligonucleotides which are better at discriminating between their homopolymeric complementary sequence and a related target sequence. In addition, the present invention provides assays which can be used (and adapted) to identify oligonucleotide modifications that destabilize mismatches.

The invention also concerns the development of primers which decrease mispriming events encountered during DNA synthesis. More

specifically, the invention concerns the development of primers containing at least one modified nucleoside, which decrease the number of internal mispriming events during cDNA generation, thereby improving the efficiency of correct priming from the *bona fide* 3' poly (A) tail.

The present invention further relates to universal primers which reduce the proportion of mismatches during genetic engineering methods such as, for example, mRNA purification, 3' RACE, 5' RACE, PCR, sequencing and the like. In a particularly preferred embodiment, the present invention relates to the incorporation of at least one universal base in an oligonucleotide comprising a homopolymeric stretch in order to reduce mismatches to its homopolymeric target sequence, and thereby generating a modified oligonucleotide. The invention concerns more particularly modified oligonucleotides, wherein a homopolymeric-stretch of the oligos contains a modification which improves their binding to their target sequence. More specifically, the present invention relates to primers or oligos incorporating at least one 3-nitropyrrole modification in the homopolymeric stretch.

The invention also concerns assays to identify modifications in oligonucleotides which reduce the proportion of mismatches and mispriming events, comprising a random or rational design of modifications of a chosen primer, a hybridization thereof with its target sequence to form a duplex, a synthesis of DNA priming from this duplex and an analysis of the synthesized DNA to assess for the presence of mispriming events, wherein the number of mispriming events produces cDNAs of truncated sizes compared to cDNAs produced by initiation from the *bona fide* priming site (i.e. the homopolymeric priming site).

In accordance with the present invention, there is therefore provided a method for destabilizing non-specific duplex formation between an oligonucleotide and a target nucleic acid, wherein at least one of the oligonucleotide and target nucleic acid comprises a homopolymeric sequence, the method comprising an incubation of the target nucleic acid with a modified

oligonucleotide, wherein the modified oligonucleotide includes a modification which decreases or abrogates hydrogen bonding between same and non-specific target sequences and thus enables a discrimination between a *bona fide* duplex formation and an artifactual one, under the conditions of hybridization used. In accordance with a preferred embodiment of the present invention, the target nucleic acid is a homopolymeric sequence.

In accordance with the present invention, there is also provided a method for increasing the proportion of full length cDNA clones in a library, comprising a use of a modified oligo d(T) primer during first strand synthesis, wherein the modification decreases or abrogates hydrogen bonding between the modified oligo d(T) primer and a non-specific target sequence, thereby increasing the proportion of full length cDNA clones.

In accordance with another aspect of the present invention, there is provided a method for reducing mispriming events during DNA synthesis, comprising a use of a modified oligonucleotide to prime the DNA synthesis, wherein the modification decreases or abrogates hydrogen bonding between the modified primer and a non-specific target sequence, thereby reducing mispriming events.

In accordance with yet another aspect of the present invention, there is provided modified oligonucleotide primers that destabilize non-specific duplex formation and reduce mispriming during DNA synthesis.

While the method of the instant invention is demonstrated during first strand cDNA synthesis to improve the quality of the cDNA population by reducing the number of clones containing aberrant 3' ends due to oligo d(T) mispairing, and more specifically using the eIF-4GII mRNA template, the present invention, which has broad utility, is not so limited. Although 3' mispairing is a general problem encountered when generating cDNA libraries from a number of organisms, this problem can be particularly exacerbated when generating cDNA libraries from organisms that have A rich genomes, since the number of internal A-rich stretches will be higher in genes from these organisms. This type

of incomplete A-tract is expected to misanneal to oligo d(T) and produce truncated cDNAs during library construction. An example of such an organism is *Plasmodium falciparum*, the parasite responsible for malaria transmission by mosquitos. Thus, the present invention provides the means to destabilize mispairing of an oligonucleotide or primer to a non-targeted or non-specific nucleic acid sequence from any organism or nucleic acid sequence-containing entity, thereby increasing the proportion of duplexes formed between the oligonucleotide or primer and its proper targeted sequence. In one preferred embodiment of the present invention, the modified primer comprises an essentially homopolymeric stretch of nucleotides (including a modification) which targets its complementary homopolymeric sequence.

While the instant invention is demonstrated using an oligo d(T)●Z primer (an oligo d(T) primer in which two of the thymine bases are substituted by 3-nitropyrrole), the instant invention is not so limited. For example, the position of the modified bases within the examplified oligonucleotide, oligo d(T) primer, can be altered (Fig. 2C) without changing the discrimination between primer and either complementary template or partially complementary template. Indeed, Guo et al. (1997, *supra*) have changed the position of 2 universal nucleosides within a given heteropolymeric oligonucleotide and shown that in many cases increased discrimination between perfect matched template and mismatched template is maintained. Thus, the instant invention extends to any homopolymeric-stretch-containing oligonucleotide (or any oligonucleotide designed to bind to a homopolymeric target sequence) such as an oligo d(T) primer containing modified nucleosides at any position, provided that such modification maintains the discriminating ability of the oligonucleotide under suitable assay conditions. It should be clear to the person of ordinary skill that the present invention further provides the means to assess whether the modifications alter this discriminating activity of the oligonucleotide. It should also be clear that any type of homopolymeric-complementary sequence duplex formation could be improved by the instant

invention. In a broad sense therefore, the present invention provides the means and methods to generate oligos or primers with improved discrimination to their complementary homopolymeric sequence compared to non-complementary sequence.

It should be clear to a person of ordinary skill that the present invention has broad implications since it demonstrates that a modification which results in destabilization of a duplex (examplified with oligo d(T), having 2 substitutions, and its poly A target sequence), significantly decreases the proportion of mismatches and of mispriming events. Hence, it is expected that other types of destabilization of the hydrogen bonds between an oligonucleotide and its target sequence would have the same effect. Non-limiting examples of modifications of the oligonucleotide which would result in such a destabilization of the duplex formation, include modifications which reduce or abrogate hydrogen bonding. Non-limiting more specific examples include known base modifications, base analogs (e.g. inosine, as exemplified hereinbelow), universal bases, and partial mismatches. Of course, it will be understood that such modifications should not favor duplex formation with a non-desired target sequence.

It should also be understood that the different modifications of the oligonucleotides encompassed by the present invention can be adapted by the person of ordinary skill to suit particular utilities (e.g. mRNA purification, sequencing).

The present invention should not be limited to the modifications of oligonucleotides with 3-nitropyrrole, since other universal bases are well known in the art. Indeed, in addition to 3-nitropyrrole, a number of universal nucleosides have been synthesized and characterized (Millican et al. 1984, *supra*; Inone et al., 1985, *supra*; Fukada et al., 1986, *supra*; Seela et al.,1986, *supra*; Eritja et al., 1986, *supra;* Habener et al., 1988, *supra*; Lin et al., 1989, *supra*; Francois et al., 1990, *supra;* Brown et al., 1991, *supra*). Other examples of universal bases can be found at

www.Synthegen.com/products/bases.html. Thus, the present invention covers any homopolymeric-stretch-containing oligo (e.g. oligo d(T) primer), containing at least one universal nucleoside which allows for enhanced discrimination when hybridizing to perfect versus mismatched templates. In addition, the demonstration that a base analog, such as inosine, inserted into the homopolymeric stretch also enhances discrimination between the target sequence and a mismatched sequence, shows that the present invention covers any homopolymeric-stretch-comprising oligo containing at least one modified base which reduces or abrogates hydrogen bonding in the sequence which is complementary to the targeted sequence..

A non-limiting example of an alternative use of this technology is in mRNA purification, by replacing oligo d(T) affinity matrixes currently employed with modified oligo d(T) according to the instant invention. An oligo d(T)•Z affinity matrix would perform the same task, except that binding to internal A-rich stretches would be minimized and could result in a purification method with a higher stringency than currently employed. This matrix, for example, could provide a better selection between eukaryotic mRNA and contaminating mycoplasmic RNA (which is A-T rich). Since mycoplasms often contaminate tissue culture cell lines, co-purification of mycoplasma RNA with eukaryotic mRNA on oligo d(T) column can produce cDNA libraires contaminated with mycoplasma clones.

Often, the sequence of a particular RNA must be interogated. Reverse transcriptase (RT), in combination with PCR, can be used to amplify a given region on an RNA template. The use of oligo d(T)•Z as primer in the RT reaction would ensure that the 3' end of the mRNA is represented on the cDNA template. Thus, the present invention can also be incorporated into current 3' RACE (Rapid Amplification of cDNA Ends) protocols, designed to obtain the 3' end of a given clone.

In some cloning protocols, first strand synthesis is followed by homopolymeric tailing of the products utilizing terminal deoxynucleotidyl

transferase. For example, dGTP can be utilized to add a homopolymeric stretch of G's at the 5' end of the cDNA. Thus the DNA polymerase utilized in second strand synthesis can take advantage of this G-stretch by priming from an oligo d(C) primer annealed to the G-stretch positioned at the 5' end. This procedure has the advantage of maintaining the sequence at the 5' terminal end of the cDNA, and is also used in 5' RACE strategies to identify the 5' end of mRNAs (Frohman et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:8998-9002; Loh et al., 1989, *Science* 243:217-220) (Fig. 5). One drawback of this approach however is that, since 5' untranslated regions of mRNAs are usually GC rich, the oligo d(C) can misprime from internal G-rich regions, producing less than full-length cDNAs. It is expected that the incorporation of universal nucleosides into such homopolymeric-stretch-containing primers to generate the modified oligos or primers of the present invention will increase the specificity of binding and generate cDNAs which terminate at the bona fide 5' end. Thus, the present invention further relates to cloning procedures or RACE protocols involving priming of second strand synthesis from a homopolymeric tail.

It may be desirable in some PCR protocols to utilize modified oligonucleotides according to the present invention, wherein the modified oligo comprises a homopolymeric stretch containing at least one universal nucleoside (or other non-specific duplex destabilizing modifications), to achieve increased discrimination between a target site (or several target sites) of interest when generating a specific product or a set of products (for example use of an oligo d(T) primer to prime DNA synthesis from the A-rich stretch of Alu repeats in humans). Since these products can be developed to be used as genetic markers (by identifying polymorphisms residing with the sequence of the product), changing the specificity of targeting by altering the specificity of the oligo d(T) primer, could result in a more consistent representation of the final PCR products. The present invention thus further relates to the use of universal oligonucleotides or other modified oligonucleotides, during PCR amplification.

DEFINITIONS

Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission.

Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for cell cultures, infection, molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference manuals such as for example Sambrook et al. (1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratories) and Ausubel et al. (1994, Current Protocols in Molecular Biology, Wiley, New York).

The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency. For certainty, it is emphasized that the present invention finds utility with nucleic acids in general. Non-limiting examples of nucleic acids which can be used in accordance with the teachings of the present invention include that from eukaryotic cells such as that of animal cells, plant cells, or microorganisms as well as that from prokaryotic cells.

As used herein, the term "homopolymeric sequences" refers to a sequence composed of a single type of nucleotide base (adenosine A; cytosine C; guanine G; thymine T; uracil U) or of a less common base (non-limiting examples including inosine, I; and pseudouridine, Ψ).

As used herein, "nucleic acid molecule", "nucleic acid sequence" or "sequence" refer to a polymer of nucleotides. Non-limiting examples thereof include DNA (e.g. genomic DNA, cDNA) and RNA molecules (e.g. mRNA). The nucleic acid molecule can be obtained by cloning techniques

or synthesized. DNA can be double-stranded or single-stranded (coding strand or non-coding strand [antisense]).

The term "recombinant DNA" as known in the art refers to a DNA molecule resulting from the joining of DNA segments. This is often referred to as genetic engineering.

The terminology "amplification pair" refers herein to a pair of oligonucleotides (oligos) of the present invention, which are selected to be used together in amplifying a selected nucleic acid sequence by one of a number of types of amplification processes, preferably a polymerase chain reaction. Other types of amplification processes include ligase chain reaction, strand displacement amplification, or nucleic acid sequence-based amplification, as explained in greater detail below. As commonly known in the art, the oligonucleotides are designed to bind to a complementary sequence under selected conditions.

The nucleic acid (e.g. DNA or RNA) for practicing the present invention may be obtained according to well known methods.

"Oligonucleotides" or "oligos" or "primers" define a nucleic acid molecule composed of nucleotides (ribo or deoxyribonucleotides). Oligonucleotide probes or primers of the present invention may be of any suitable length, depending on the particular assay format and the particular needs and targeted genomes employed. In general, the oligonucleotide probes or primers are at least 10 nucleotides in length, preferably below 50 nucleotides. Preferably, the oligos or primers have lengths between 15 and 40 nucleotides, more preferably between 20 to 30 nucleotides. Of course, the probes or primers of the present invention may be adapted to be especially suited to a chosen nucleic acid amplification system. As commonly known in the art, the oligonucleotide probes and primers can be designed by taking into consideration the melting point of hydrizidation thereof with its targeted sequence (see below and in Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, CSH Laboratories; Ausubel et al., 1989, in Current Protocols in

Molecular Biology, John Wiley & Sons Inc., N.Y.). The size of the oligonucleotide will be dictated by the particular situation and ultimately on the particular use thereof and adapted accordingly by the person of ordinary skill. An oligonucleotide can be synthesized chemically or derived by cloning according to well known methods. For example, the skilled artisan will be able to adapt the length of the essentially homopolymeric stretch-containing oligo (the targeting stretch wherein the homopolymeric stretch has been modified in accordance with the teachings of the present invention), to particular needs, as a function of the targeted stretch and other parameters such as the sequence of the duplex, the conditions of the assay (and hence of the Tm) and the presence of additional sequences, flanking the essentially homopolymeric stretch (at the 5' and/or 3' end thereof).

The term "oligonucleotide" or "DNA" molecule or sequence refers to a molecule comprised of the deoxyribonucleotides adenine (A), guanine (G), thymine (T) and/or cytosine (C), in a double-stranded form, and comprises or includes a "regulatory element" according to the present invention, as the term is defined herein. The term "oligonucleotide" or "DNA" can be found in linear DNA molecules or fragments, viruses, plasmids, vectors, chromosomes or synthetically derived DNA. As used herein, particular double-stranded DNA sequences may be described according to the normal convention of giving only the sequence in the 5' to 3' direction.

Probes and oligonucleotides of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and α-nucleotides and the like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic acid molecule. Acids Res., 14:5019. Probes of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and preferably of DNA. General teachings on the synthesis of oligonucleotides and substituents and modifications thereof can be found for example in US

5,438,131. The selection of the best suited synthesis pathway of an oligonucleotide and of the appropriate modifications, and substituents to be used, may be selected accordingly by the person of ordinary skill to which the instant invention pertains.

The modified oligonucleotides of the present invention can be synthesized chemically or produced through recombinant DNA technology. All these methods are well known in the art. According to the present invention, the modified oligonucleotides are molecules comprising an essentially homopolymeric stretch or sequence composed of a single type of nucleotide and at least one type of modification which enables a destabilization of mismatches. In a preferred embodiment, these modified oligonucleotides are a molecule composed of a single type of nucleotide (ribo- or deoxyribonucleotides, A, C, G, T or U) and containing at least one universal nucleoside. As mentioned above, the length is between 10 and 50 nucleotides. Of course, it should be recognized that in a case where more than one modification or nucleotide which destabilizes mismatches is used, it need not be the same type of "modification". In some embodiments of the present invention, the modified oligonucleotides of the present invention comprise an essentially homopolymeric stretch and a "3' lock" (see below) or a sequence enabling the creation of a restriction site.

As used herein, a "primer" defines an oligonucleotide which is capable of annealing to a target sequence, thereby creating a double stranded region or duplex which can serve as an initiation point for DNA synthesis under suitable conditions.

"Nucleic acid hybridization" refers generally to the hybridization of two single-stranded nucleic acid molecules having complementary base sequences, which under appropriate conditions will form a thermodynamically favored double-stranded structure. Examples of hybridization conditions can be found in the two laboratory manuals referred above (Sambrook et al., 1989, *supra* and Ausubel et al., 1989, *supra*) and are commonly known in the art. In the case of a hybridization to a nitrocellulose

filter, as for example in the well known Southern blotting procedure, a nitrocellulose filter can be incubated overnight at 65°C with a labeled probe in a solution containing 50% formamide, high salt (5 x SSC or 5 x SSPE), 5 x Denhardt's solution, 1% SDS, and 100 µg/ml denatured carrier DNA (e.g. salmon sperm DNA). The non-specifically binding probe can then be washed off the filter by several washes in 0.2 x SSC/0.1% SDS at a temperature which is selected in view of the desired stringency: room temperature (low stringency), 42°C (moderate stringency) or 65°C (high stringency). The selected temperature is based on the melting temperature (Tm) of the DNA hybrid. Of course, RNA-DNA hybrids can also be formed and detected. In such cases, the conditions of hybridization and washing can be adapted according to well known methods by the person of ordinary skill. Stringent conditions will be preferably used (Sambrook et al.,1989, *supra*).

The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection).

Probes or oligonucleotides can be labeled according to numerous well known methods (Sambrook et al., 1989, supra). Non-limiting examples of labels include ^{3}H, ^{14}C, ^{32}P, and ^{35}S. Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other detectable markers for use with probes, which can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

As commonly known, radioactive nucleotides can be incorporated into probes of the invention by several methods. Non-limiting examples thereof include kinasing the 5' ends of the probes using gamma ^{32}P ATP and polynucleotide kinase, using the Klenow fragment of Pol I of *E. coli* or reverse transcriptase in the presence of radioactive dNTP (e.g. uniformly labeled

DNA probe using random oligonucleotide primers in low-melt gels), using the SP6/T7 system to transcribe a DNA segment in the presence of one or more radioactive NTP, and the like.

Amplification of a selected, or target, nucleic acid sequence may be carried out by a number of suitable methods. See generally Kwoh et al., 1990, Am. Biotechnol. Lab. 8:14-25. Numerous amplification techniques have been described and can be readily adapted to suit particular needs of a person of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-based amplification, the Qβ replicase system and NASBA (Kwoh et al., 1989, Proc. Natl. Acad. Sci. USA 86, 1173-1177; Lizardi et al., 1988, BioTechnology 6:1197-1202; Malek et al., 1994, Methods Mol. Biol., 28:253-260; and Sambrook et al., 1989, *supra*). Preferably, amplification will be carried out using PCR.

Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosures of all three U.S. Patent are incorporated herein by reference). In general, PCR involves, a treatment of a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) under hybridizing conditions, with one oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of each primer which is synthesized is complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products using the same primers. Following a sufficient number of rounds of synthesis of extension products, the sample is analysed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization following EtBr staining of the DNA following gel electrophores, or using a detectable label in accordance with known techniques,

and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al. Eds, Acad. Press, 1990).

Ligase chain reaction (LCR) is carried out in accordance with known techniques (Weiss, 1991, Science 254:1292). Adaptation of the protocol to meet the desired needs can be carried out by a person of ordinary skill. Strand displacement amplification (SDA) is also carried out in accordance with known techniques or adaptations thereof to meet the particular needs (Walker et al., 1992, Proc. Natl. Acad. Sci. USA 89:392-396; and ibid., 1992, Nucleic Acids Res. 20:1691-1696).

As used herein, the term "gene" is well known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise to a specific polypeptide or protein. It will be readily recognized by the person of ordinary skill, that the nucleic acid sequence of the present invention can be incorporated into anyone of numerous established kit formats which are well known in the art.

The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

The term "allele" defines an alternative form of a gene which occupies a given locus on a chromosome.

As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter cell. As well known, a mutation can be, for example, a detectable change in one or more deoxyribonucleotide. For example, nucleotides can be added, deleted, substituted for, inverted, or transposed to a new position. Spontaneous mutations and experimentally induced mutations exist. The result of a mutations

of nucleic acid molecule is a mutant nucleic acid molecule. A mutant polypeptide can be encoded from this mutant nucleic acid molecule.

As used herein, the term "purified" refers to a molecule having been separated from a cellular component. Thus, for example, a "purified protein" has been purified to a level not found in nature. A "substantially pure" molecule is a molecule that is lacking in most other cellular components.

The present invention also relates to a kit comprising the oligonucleotide primers of the present invention. For example, a compartmentalized kit in accordance with the present invention includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allow the efficient transfer of reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample (DNA, RNA or cells), a container which contains the primers used in the assay, containers which contain enzymes, containers which contain wash reagents, and containers which contain the reagents used to detect or isolate the extension products.

Of course, cDNA cloning kits could be adapted by inserting thereinto the primers of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1 (PRIOR ART) shows an example of the steps involved in generating cDNA libraries from mRNA. Although a number of strategies can be used for cDNA library generation, of which only one is shown, all libraries require as a first step, a primer from which the reverse transcriptase

(RT) can prime. In the case of full-length cDNA libraries, an oligo d(T) primer is used because it anneals to the 3' poly (A) tail of the eukaryotic mRNAs. In the case of prokaryotic, some viral, or other eukaryotic mRNAs which lack a poly (A) tail, a homopolymeric stretch of nucleoside 5'-monophosphates can be added to the 3' end of the mRNA. For example, poly (A) polymerase can be used to add a poly (A) tail to mRNAs which lack one. An oligonucleotide which contains complementary nucleotides (e.g. oligo d(T)) is then annealed to the mRNA and serves as primer for the RT.

Figure 2A shows a hybridization of oligo $d(T)_{15}$ primer to the *bona fide* poly (A) tail of an mRNA (right) or to an internal A-rich stretch (left) within the mRNA by conventional oligo d(T) primer used in current cDNA library construction. Although the length of the primer used can differ, and the two 3' most nucleotides are sometimes (A,C,G,T) and (A,G,C) to "lock" the oligonucleotide in place at the junction of the body of the mRNA and the poly (A) tail, neither of these modifications prevent the misannealing of the oligo d(T) primer to internal A-rich stretches. The asterisks denotes mispairing resulting in destabilization of the duplex. Figure 2B shows the chemical structure of 3-nitropyrrole. Figure 2C shows the structure of oligo d(T)●Z primer. Figure 2D shows the expected discrimination between the poly (A) tail (right) and internal A-rich stretches (left) when hybridizing to oligo d(T)●Z. The asterisks denote mispairing resulting in destabilization of the duplex and circles represent 3-nitropyrrole artificial mismatches.

Figure 3A shows the structure of the eIF-4GII cDNA construct used to analyze mispriming at the 3' end. The location of four internal A-rich sequences are shown - all of which generated 3' truncated clones when eIF-4GII was isolated from a cDNA library. The plasmid was linearized with Asp 718 and T7 RNA polymerase used to generate a ~2400 nt ^{3}H-test transcript. Figure 3B shows the integrity of the *in vitro* generated transcript following fractionation on a formaldehyde 1.2% agarose gel, treatment with EN^{3}HANCE, and autoradiography of the dried gel. Figure 3C shows the alkaline agarose analysis

of RT products generated by priming synthesis with oligo d(T) (lane 1) or oligo d(T)•Z (lane 2) using MMLV RT. Complementary DNA was labeled with α-^{32}P-dCTP. The position of migration of truncated products are indicated by a filled circle and full length product by an arrow. These results directly demonstrate correction of 3' mispriming by utilizing oligo d(T)•Z as primer during first strand synthesis.

Figure 4A shows the structure of eIF-4GII construct used to demonstrate mispriming at the 3' end. The location of five oligonucleotides (a, b, c, d, e) used in the hybridization assay to map the sites of 3' mispriming by oligo d(T) are shown. The nucleotide targets of the oligonucleotides on eIF-4GII are:

Oligo a, ^{5567}GAAATTGACTCAGTACTATT^{5584};

Oligo b, ^{5416}GAAGGAAATGCTGTGGACC^{5535};

Oligo c, ^{5194}TGTATAATAGAAAAGCAGAG^{5213};

Oligo d, ^{5068}TTTTAAACAAGGACTCATAC^{5087}; and

Oligo e, ^{4781}AAGAGGAGTCTGAGGATAAC^{4800}.

Figure 4B shows the Southern blot of the alkaline agarose gel of RT products generated by priming synthesis with either oligo d(T) or oligo d(T)•Z. Marker lane refers to the 1 kb size ladder from GIBCO and sizes (in bp) are indicated to the left of the diagram. eIF-4GII DNA refers to a DNA fragment of eIF-4GII used as a positive control for DNA hybridizations. Oligonucleotides used as probes on each blot are indicated below each panel. The asterisks on the left denotes the cDNA product obtained by priming at the correct poly (A) site. The filled circle denotes the cDNA product obtained by priming from the A-rich stretch between nucleotides 5550-5575, whereas the arrow denoted the cDNA obtained by priming from nucleotides 5085-5120.

Figure 5 shows an example illustrating mispriming events at the 5' end of cDNAs during cDNA library construction of 5' RACE analysis to extend the sequence of known genes.

Figure 6 shows an autoradiograph following oligo d(T) primed first strand synthesis on eIF-4GII mRNA. Lane 2 oligo d(T) control; lane 3 oligo d(T)●Z; and lane 4 oligo d(T)●I. A molecular mass standard ladder is shown in lane 1.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The demonstration of the destabilization effect of non-specific or artifactual duplex formation and of its concurrent effect on mismatch and/or mispriming events was carried out with an oligo d(T) primer, modified with one or two universal analogues. Whether such an introduction could result in increased discrimination between the perfectly matched target of that primer (i.e. the 3' poly (A) tail of the mRNA) and an imperfect matched sequenced (internal A-rich stretches) was analyzed.

More specifically, an oligo d(T) primer, called oligo d(T)●Z was generated, in which two of the thymine bases were substituted by 3-nitropyrrole (Fig. 2C). General teachings on 3-nitropyrrole, the synthesis thereof and the like can be found for example in U.S. 5,438,131.

To test whether this primer can reduce mispriming from internal A-rich sequences (Fig. 2D), a cDNA clone from eIF-4GII, a eukaryotic translation factor, was obtained. When cDNA clones to this gene were initially isolated, only one of 5 clones had the correct 3' end. Sequence characterization of these clones demonstrated that all the truncated clones were the result of internal priming by oligo d(T) at four different sites (denoted as leftward arrows in Fig. 3A). *In vitro* transcribed RNA generated from this clone thus serves as an excellent test reagent to determine the ability of the 3-nitropyrrole substituted

oligo d(T) to decrease the number of mispriming events. The quality of the *in vitro* transcribed RNA is shown in Fig. 3B and demonstrates that the test template is intact. This RNA was then annealed to oligo d(T) or oligo d(T)•Z, and reverse transcription performed with MMLV RT. As shown in Fig. 3C, use of oligo d(T) on this template resulted in shorter than full-length products (>95%) generated as a result of internal priming (Fig. 3C, lane 1). However, use of oligo d(T)•Z as primer on the same template resulted in the majority (>95%) of products being full-length (Fig. 3C, lane 2).

These results demonstrate that use of oligo d(T)•Z in reverse transcription reactions significantly improves the specificity for the 3' poly (A) tail and demonstrates the usefulness of this procedure in destabilizing non-specific duplex formation and more particularly for generating full length cDNAs.

The sites of mispriming with oligo d(T) on the control eIF-4GII template were identified (Fig. 4). This was done by fractionating the products of RT reactions performed with either oligo d(T) or oligo d(T)•Z on an alkaline agarose gel followed by transfer to a nylon membrane. This membrane was then probed, by hybridization, with oligonucleotides designed to target various regions of the 3' untranslated region of eIF-4GII (oligonucleotides are labelled a, b, c, d and e in Fig. 4A). As shown in Fig. 4B, hybridization with oligonucleotide "a" detected correctly primed cDNA when both oligo d(T) and oligo d(T)•Z were used as primer. Hybridization with oligonucleotides b and c, detected a novel truncated product when the RT reaction was primed with oligo d(T), indicating mispriming from an internal A-rich stretch with this primer (Fig. 4B). Hybridization with oligonucleotides d and e, detected an additional novel, more abundant truncated product (denoted by arrowheads in Fig. 4B) when the RT reaction was primed with oligo d(T), indicating mispriming from a second internal A-rich stretch with this primer but not with oligo d(T)•Z (Fig. 4B).

Mispriming event are common in Rapid Amplification of cDNA ends (RACE). An example of mispriming at the 5' end of cDNAs during 5' RACE analysis is shown in Figure 5. Such mispriming events could be resolved by

incorporating a universal nucleoside into the oligo d(C) primer to increase the discrimination between the homologous target (e.g. - the 5' end G tail) and an internal G-rich sequence. It is expected that incorporation of at least one universal base (e.g. 3-nitropyrrole) in the homopolymeric oligo d(C) primer should significantly reduce such mispriming.

The present invention is illustrated in further detail by the following non-limiting example.

EXAMPLE 1

Destabilization of mispriming and reduction of mispriming using an oligo d(T)•I primer

To demonstrate that other "modifications" of nucleotides that destabilize hydrogen bonding between mismatched sequences could be used in accordance with the present invention, the oligo d(T) primer was modified by inserting thereinto deoxynucleotide deoxyinosine (I).

An oligonucleotide [called oligo d(T)•I] having the sequence 5'TTTTTTTI*TTTTTTTTTI*TTTTT3' was thus synthesized (McGill University Sheldon Biotechnology Center), where I* represents the position where the 2'deoxyinosine was incorporated into the oligonucleotide. Reverse transcription reactions were performed on *in vitro* generated eIF-4G mRNA templates (1 µg) with Superscript II™ (LifeTechnologies) under conditions recommended by LifeTechnologies. Oligonucleotide primers that were utilized to prime the first strand synthesis were 0.1 µg of either Oligo $d(T)_{15}$, oligo d(T)•Z, or oligo d(T)•I. The radioisotope α-^{32}P-dCTP (New England Nuclear) was used as a tracer to monitor the quality of the cDNA product. Following the generation of cDNA products at 42°C for 1 hr, the mixture was extracted with phenol/chloroform, back extracted with an equal volume of water, passed through a G50™ spun column, and precipitated with 2M ammonium acetate and 2.5 volumes of ethanol. The precipitate was washed with 70% ethanol, dried and resuspended in 20 µl of water. An aliquot (5 ul) was loaded onto a 1.2% alkaline agarose gel

and electrophoresis performed at 78 volts for 6.5 hours. The gel was neutralized in 7% trichloroacetic acid for 30 minutes, dried, and exposed to X-OMAT X-ray film (Kodak) at –70°C for 10 hrs with an intensifying screen.

An photograph of the autoradiograph is presented in Figure 6. A molecular mass standard ladder is shown in lane 1 (purchased from LifeTechnologies). The cDNA product obtained by priming with oligo $d(T)_{15}$ is shown in lane 2. Clearly, the major cDNA product is shorter than full-length and arises due to internal mispriming at an internal A-rich site. As shown previously, priming with oligo d(T)•Z is able to correct the mispriming phenomenon, and in this particular experiment over 50% of the cDNA is correctly primed from the poly (A) tail of the mRNA (lane 3, full-length product indicated with an arrow). Priming the cDNA reaction with oligo d(T)•I also efficiently corrected the mispriming reaction observed with oligo $d(T)_{15}$ primer and resulted in a significant proportion of cDNAs being full-length (lane 4, full length product indicated with an arrow).

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

15. The method according to claim 14, wherein said enzyme is a reverse transcriptase selected from the group consisting of avian myoblastoid virus reverse transcriptase, murine moloney leukemia virus reverse transcriptase, and human immuno deficiency virus reverse transcriptase.

16. A kit for the synthesis of cDNA, said kit comprising a modified oligo d(T) primer, wherein said modified oligonucleotide includes a modification which decreases or abrogates hydrogen bonding between same and non-specific target sequences.

17. A method for reducing mispriming events during DNA synthesis comprising a use of a modified oligonucleotide to prime said DNA synthesis, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between said modified oligonucleotide and a non-specific target sequence, thereby reducing mispriming events, while maintaining a formation of a duplex with a *bona fide* homopolymeric target sequence.

18. The method of claim 17, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

19. The method of claim 18, wherein said universal base is 3-nitropyrrole.

20. The method of claims 17, 18 or 19, wherein said oligonucleotide is a homopolymer.

WHAT IS CLAIMED IS:

1. A method for destabilizing non-specific duplex formation between an oligonucleotide and a target nucleic acid, comprising an incubation of said target nucleic acid with a modified oligonucleotide, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between said modified oligonucleotide and non-specific target sequences.

2. The method of claim 1, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

3. The method of claim 2, wherein said universal base is 3-nitropyrrole.

4. The method of one of claims 1-3, wherein said oligonucleotide is a homopolymer comprising at least one nucleotide modification.

5. A method for increasing the proportion of full length cDNA clones in a library, comprising a use of a modified oligo d(T) during first strand synthesis, wherein said modified oligo d(T) comprises a modification which decreases or abrogates hydrogen bonding between said modified oligo d(T) and a non-specific target sequence, thereby increasing the proportion of full length cDNA clones.

6. The method of claim 5, wherein said modification is at least one universal base incorporated into said oligo d(T).

7. The method of claim 6, wherein said universal base is 3-nitropyrrole.

8. The method of claim 5, wherein said modification is at least one chemically modified nucleoside incorporated into said oligo d(T).

9. The method of claim 5, wherein said modification is at least one base analog incorporated into said oligo d(T).

10. The method of claim 9, wherein said base analog is inosine.

11. The method of claim 5, wherein said modification is at least one mismatch incorporated into said oligo d(T).

12. The method of claim 5, wherein said modification is a phosphate or ribose modification incorporated into said oligo d(T).

13. The method according to one of claims 5 to 12, wherein an enzyme capable of RNA-dependent DNA polymerization is used for said first strand synthesis.

14. The method according to one of claims 5 to 12, wherein an enzyme capable of RNA-dependent RNA polymerization is used for said first strand synthesis.

21. A method for reducing mispriming during 5' RACE comprising a use of a modified oligonucleotide to prime said 5' RACE, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between said modified oligonucleotide and a non-specific target sequence, thereby reducing mispriming events while maintaining a formation of a duplex with a *bona fide* homopolymeric target sequence.

22. The method of claim 21, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

23. The method of claim 21, wherein said universal base is 3-nitropyrrole.

24. The method of claim 21, wherein said modification is at least one chemically modified nucleoside incorporated into said homopolymeric sequence.

25. The method of claim 21, wherein said modification is at least one base analog incorporated into said homopolymeric sequence.

26. The method of claim 25, wherein said base analog is inosine.

27. The method of claim 21, wherein said modification is at least one mismatch incorporated into said homopolymeric sequence.

28. The method of claim 21, wherein said modification is a phosphate or ribose modification destabilizing mismatch recognition incorporated into said homopolymeric sequence.

29. A kit for 5' RACE comprising a modified oligonucleotide primer, comprising a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between said modified oligonucleotide and non-specific target sequences.

30. A method for reducing mispriming during 3' RACE comprising a priming of said 3' RACE with a modified oligonucleotide, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between said modified oligonucleotide and a non-specific target sequence, thereby reducing mispriming events while maintaining a formation of a duplex with a *bona fide* homopolymeric target sequence.

31. The method of claim 30, wherein said modification is at least one universal base incorporated into said homopolymeric sequence.

32. A method for generating *bona fide* genetic markers comprising a use of a modified oligonucleotide to prime from homopolymeric stretches, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between said modified oligonucleotide and non-specific target sequences.

33. The method of claim 32, wherein said modified oligonucleotide primes from an internal A-rich region in an Alu repeat.

34. A method for stabilizing duplex formation between an oligonucleotide and a target homopolymeric sequence comprising an incubation of said target homopolymeric sequence with a modified oligonucleotide, wherein said modified oligonucleotide comprises a homopolymeric tract having a modification which decreases or abrogates hydrogen bonding between same and non-specific target sequences.

35. A method for reducing mispriming during sequencing comprising a use of a modified oligonucleotide to prime DNA synthesis, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between same and non-specific target sequences.

36. A method to improve the discrimination between a binding of an oligonucleotide sequence to its targetted homopolymeric sequence versus a non-homopolymeric tract comprising an insertion into a homopolymeric tract of said oligonucleotide sequence of at least one modification which decreases or abrogates hydrogen bonding between same and said non-homopolymeric tract.

37. A method for increasing the proportion of full length cDNA clones in a library, comprising a use of a modified oligonucleotide during second strand synthesis from a 3' end-tailed first strand product, wherein said modified oligonucleotide comprises a homopolymeric sequence having a modification which decreases or abrogates hydrogen bonding between said

modified oligo and a non-specific target sequence, thereby increasing the proportion of full length cDNA clones.

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(71) Applicant *(for all designated States except US)*: MCGILL UNIVERSITY [CA/CA]; 3550 University Street, Montreal, Quebec H3A 2A7 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PELLETIER, Jerry [CA/CA]; 8 Lakeview, Baie D'Urfée, Quebec H9X 3B1 (CA). DAS, Manjula [IN/CA]; Apartment #205, 3484 Stanley, Montreal, Quebec H3A 1S1 (CA).

(74) Agents: DUBUC, Jean et al.; Goudreau Gage Dubuc & Martineau Walker, The Stock Exchange Tower, Suite 3400, 800 Place Victoria, P.O. Box 242, Montreal, Quebec H4Z 1E9 (CA).

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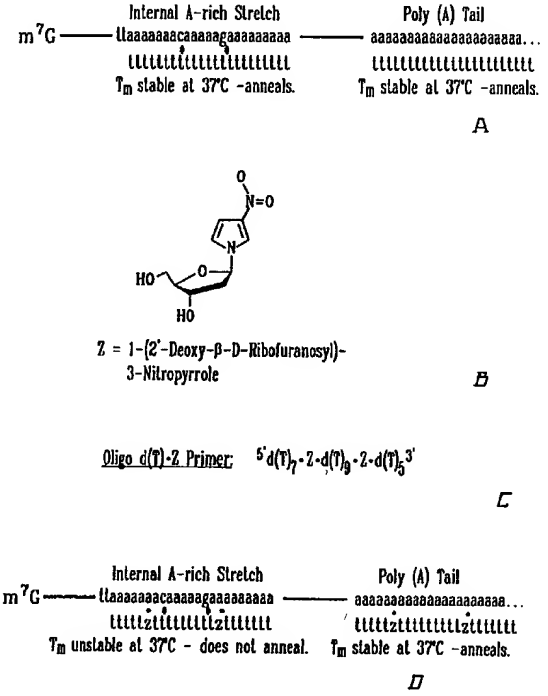
(57) Abstract

The present invention relates to the demonstration that a modification of a homopolymeric stretch in an oligonucleotide, or primer, improves the discrimination for binding of such a modified oligonucleotide or primer to its complementary homopolymeric target sequence, as compared to a non-homopolymeric sequence. More specifically, an oligo d(T) primer having two of the thymine bases substituted by 3-nitropyrrole were used in a poly A primed cDNA synthesis experiment to demonstrate an improvement in discrimination between the priming of cDNA synthesis from *bona fide* poly A sequence as compared to internal A-rich sequences. The present invention relates to modifications of homopolymeric sequences in oligos, decreasing the ridging bonding capacity, in general, since other modifications, such as an oligo d(T) primer substituted with 2' deoxyinosine was also shown to improve the discrimination between the binding to a *bona fide* poly A tail as compared to A-rich sequences. The present invention thus relates to universal primers which reduce mispriming during cDNA library construction, thereby increasing the proportion of cDNA clones having been primed from the *bona fide* 3' poly A tail. The present invention further relates to the use of the discriminating oligonucleotides of the present invention in other methods such as mRNA purification, PCR-based detection methods and sequencing.



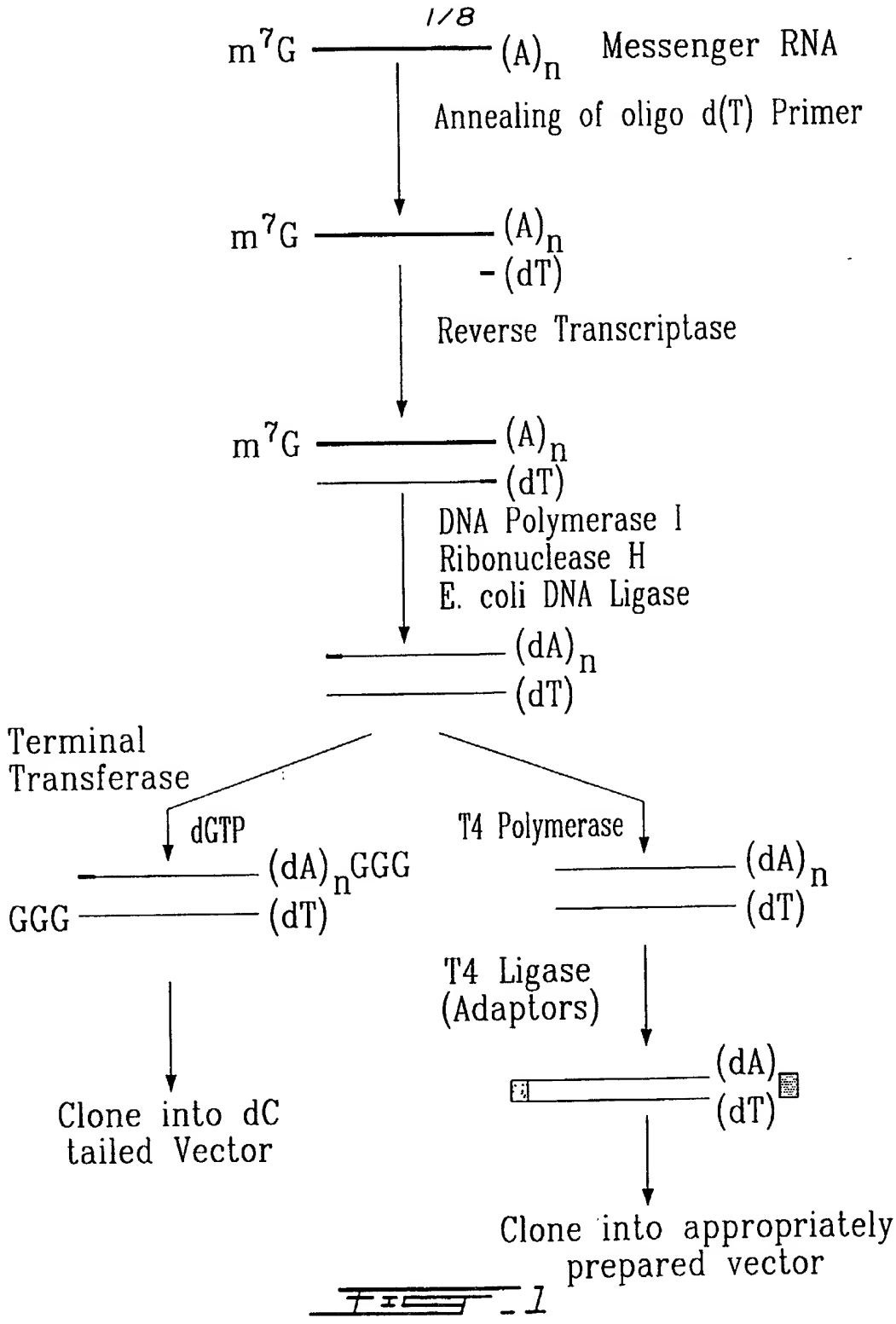

1/8
m7G (A)n Messenger RNA
Annealing of oligo d(T) Primer
m7G (A)n
(dT)
Reverse Transcriptase
m7G (A)n
(dT)
DNA Polymerase I
Ribonuclease H
E. coli DNA Ligase
(dA)n
(dT)
Terminal Transferase
dGTP
(dA)nGGG
GGG (dT)
Clone into dC tailed Vector
T4 Polymerase
(dA)n
(dT)
T4 Ligase (Adaptors)
(dA)
(dT)
Clone into appropriately prepared vector

Fig - 1

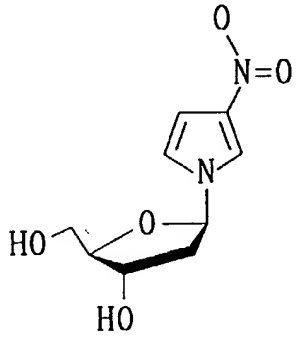
Internal A-rich Stretch — Poly (A) Tail

m^7G —— ttaaaaaaacaaaaagaaaaaaaaa —— aaaaaaaaaaaaaaaaaaaa...

ttttttttttttttttttttt ttttttttttttttttttttt

T_m stable at 37°C -anneals. T_m stable at 37°C -anneals.

Fig - 2A



Z = 1-(2'-Deoxy-β-D-Ribofuranosyl)-
3-Nitropyrrole

Fig - 2B

Oligo d(T)•Z Primer: $5'd(T)_7 \cdot Z \cdot d(T)_9 \cdot Z \cdot d(T)_5 3'$

Fig - 2C

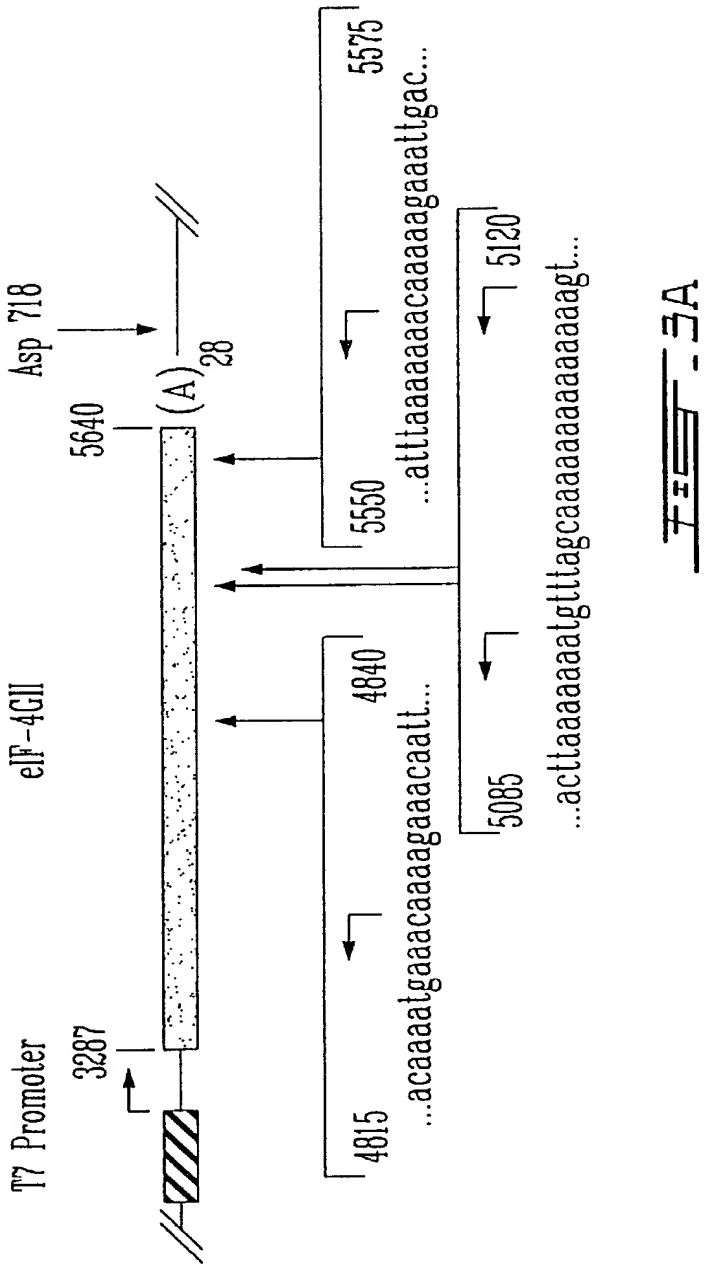
Internal A-rich Stretch — Poly (A) Tail

m^7G —— ttaaaaaaacaaaaagaaaaaaaaa —— aaaaaaaaaaaaaaaaaaaa...

ttttzttttttttztttttt ttttzttttttttztttttt

T_m unstable at 37°C - does not anneal. T_m stable at 37°C -anneals.

Fig - 2D


T7 Promoter
3287
eIF-4GII
5640
Asp 718
(A)28
4815
...acaaaatgaaacaaaagaaacaatt...
4840
5550
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5575
5085
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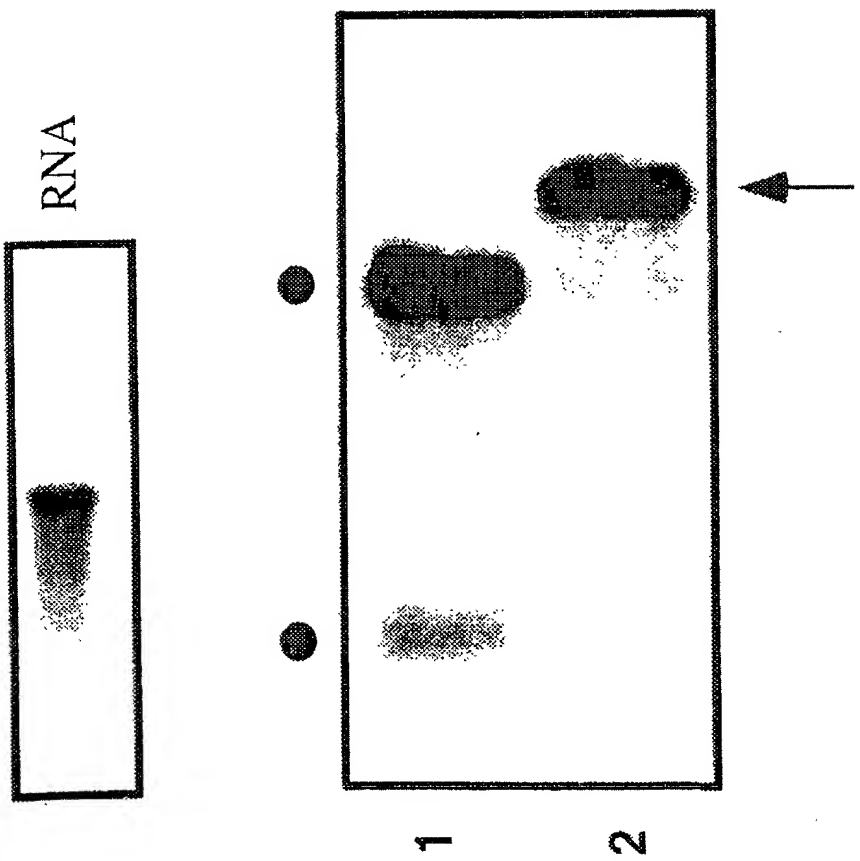
5120

FIG - 3A

Fig - 3B

Fig - 3C



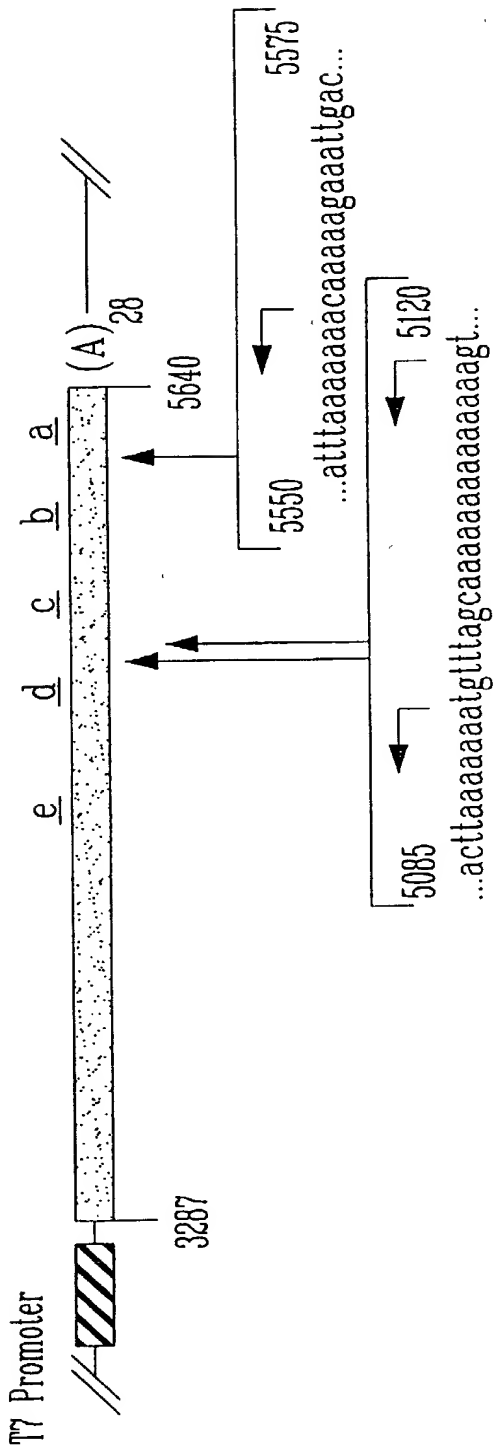

T7 Promoter
eIF-4GII
e
d
c
b
a
(A)28
3287
5640
5550
5575
...atttaaaaaaacaaaaagaaattgac...
5120
5085
...acttaaaaaaatgtttagcaaaaaaaaaaaaagt...

FIG - 4A

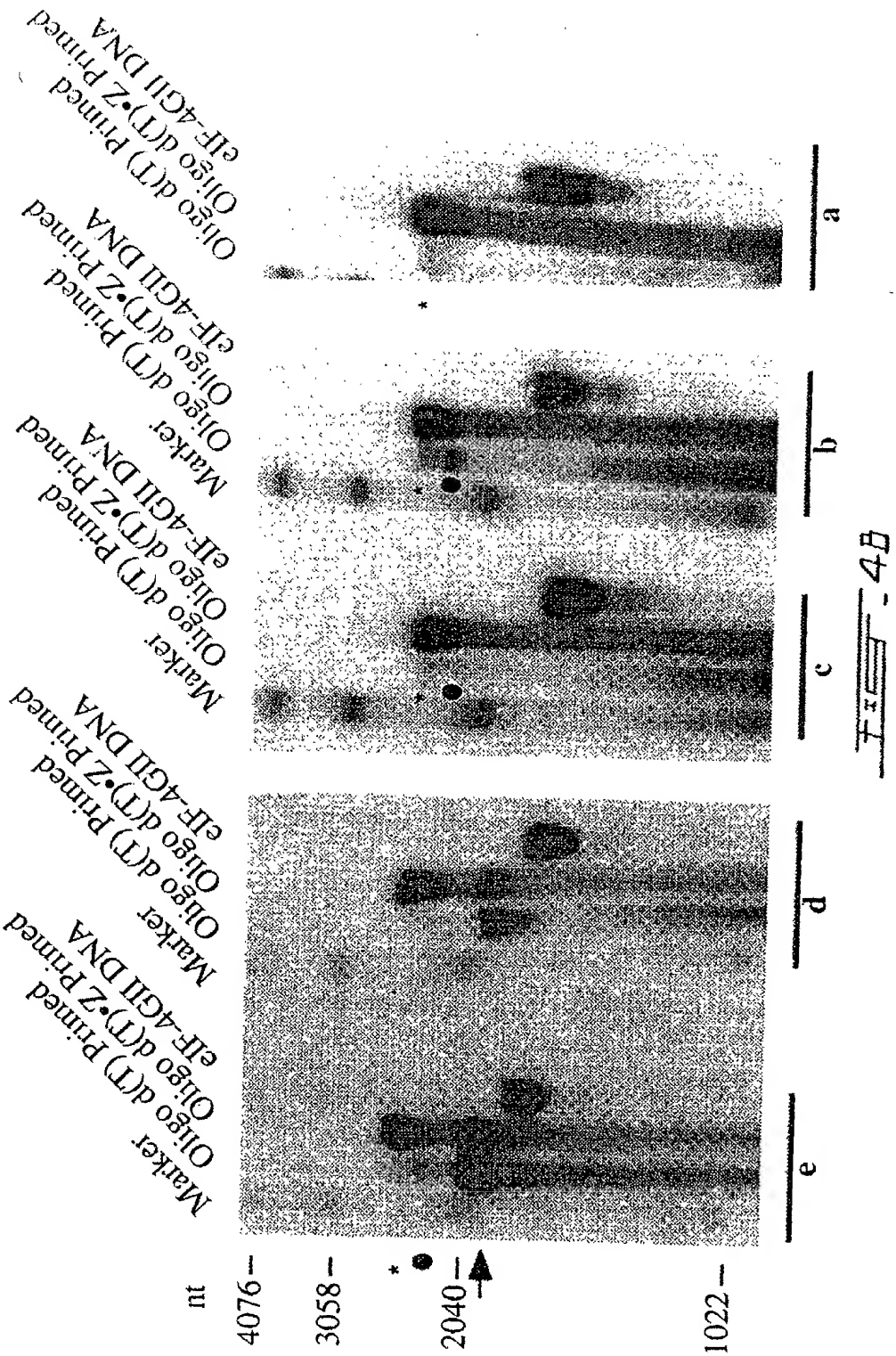

nt
4076
3058
2040
1022
a
b
c
d
e
Oligo d(T) primed eIF-4GII DNA
Marker
Oligo d(T)•Z primed eIF-4GII DNA
Oligo d(T) primed eIF-4GII DNA
Marker
Oligo d(T)•Z primed eIF-4GII DNA
Oligo d(T) primed eIF-4GII DNA
Marker
Oligo d(T)•Z primed eIF-4GII DNA

Fig-4B

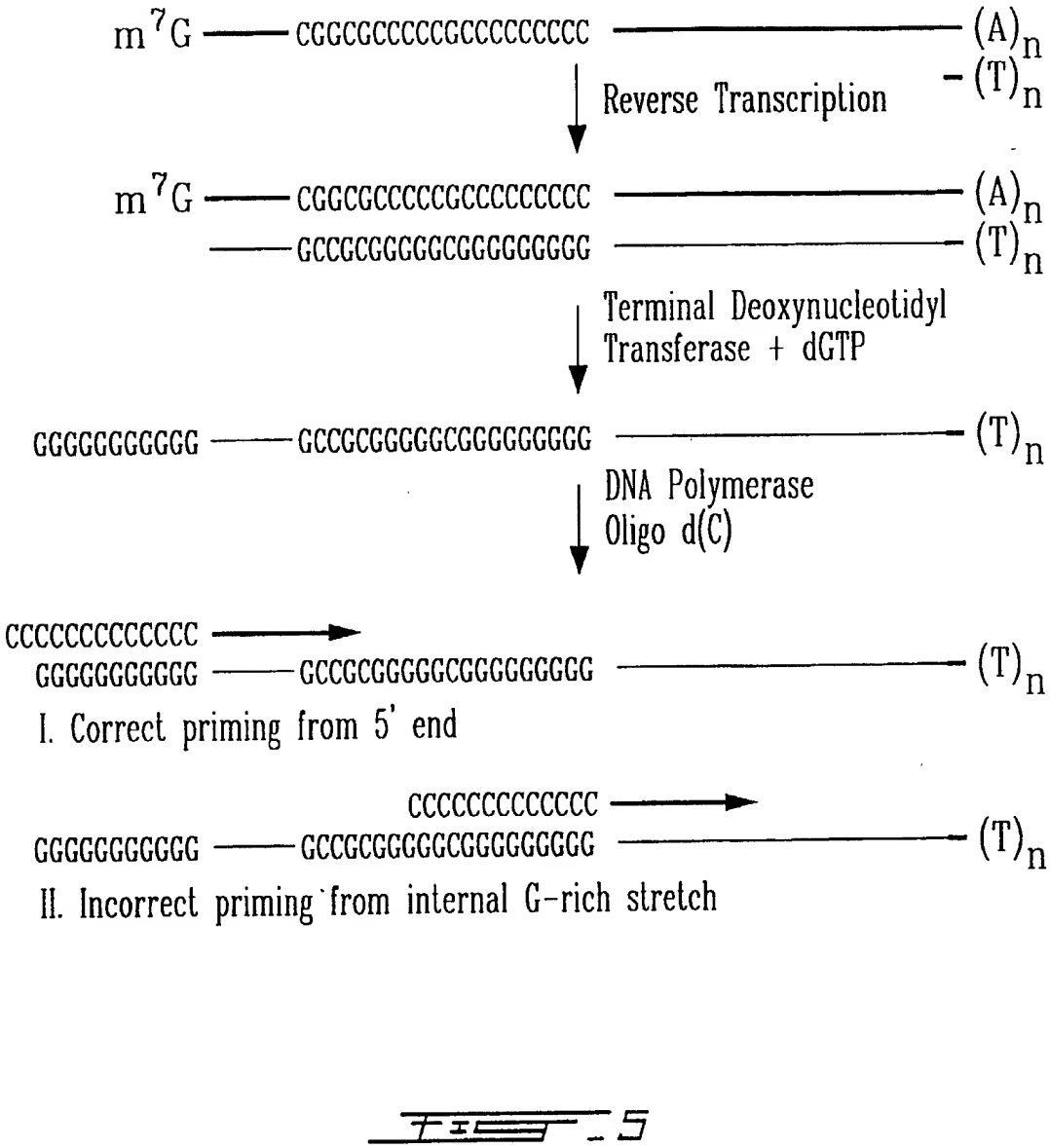

m7G — CGGCGCCCCCGCCCCCCCC — (A)n
Reverse Transcription
- (T)n
m7G — CGGCGCCCCCGCCCCCCCC — (A)n
— GCCGCGGGGGCGGGGGGGG — (T)n
Terminal Deoxynucleotidyl
Transferase + dGTP
GGGGGGGGGG — GCCGCGGGGGCGGGGGGGG — (T)n
DNA Polymerase
Oligo d(C)
CCCCCCCCCCCC
GGGGGGGGGG — GCCGCGGGGGCGGGGGGGG — (T)n
I. Correct priming from 5' end
CCCCCCCCCCCC
GGGGGGGGGG — GCCGCGGGGGCGGGGGGGG — (T)n
II. Incorrect priming from internal G-rich stretch

FIG. 5

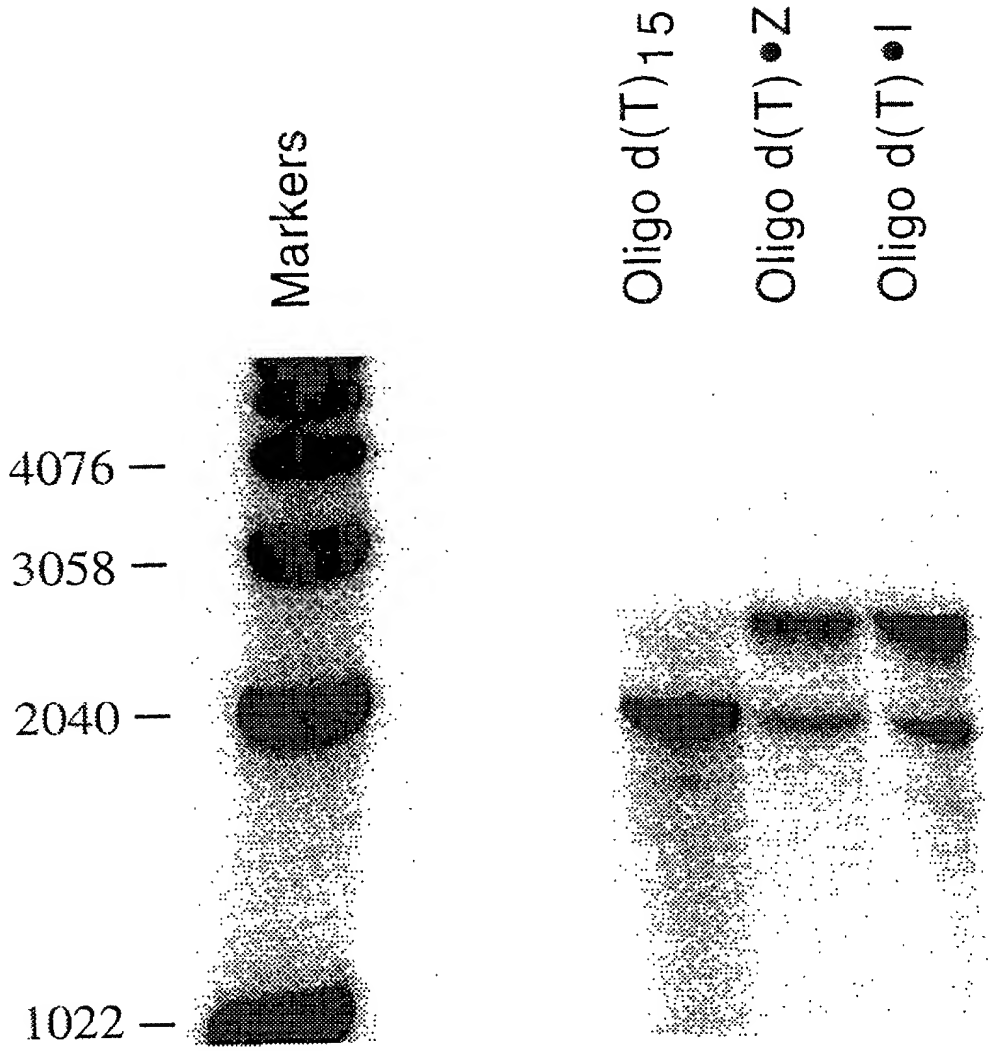


Fig. 6

PATENT
Docket No. 514012000100

DECLARATION FOR PATENT APPLICATION (Includes Reference to PCT International Applications)	ATTORNEY'S DOCKET NUMBER 514012000100

As a below named inventor I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am an original and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

OLIGONUCLEOTIDE PRIMERS THAT DESTABILIZE NON-SPECIFIC DUPLEX FORMATION AND USES THEREOF

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application

Serial No To Be Assigned
on
and was amended on (if applicable).

☒ was filed as PCT international application

Number PCT/CA99/00933
on October 6, 1999,
and was amended under PCT Article 19
on (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37 Code of Federal Regulations § 1 56(a) and (b)

I hereby claim foreign priority benefits under Title 35 United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. § 119:

COUNTRY (if PCT indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U S.C. § 119
CANADA	2,246,623	7 OCTOBER 1998	☒ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO

pa-581957

[redacted]

Declaration for Patent Application (Continued) (Includes Reference to PCT International Applications)	ATTORNEY'S DOCKET NUMBER 514012000100

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. § 120:

U.S. APPLICATIONS		STATUS *(Check one)*		
U S APPLICATION NUMBER	U S FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATIONS DESIGNATING THE U.S.			STATUS *(Check one)*		
PCT APPLICATION NUMBER	PCT FILING DATE	U S SERIAL NUMBERS ASSIGNED *(if any)*	PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. ***(List name and registration number)***

Send correspondence to: Gladys H. Monroy
Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, California 94304-1018

Direct telephone calls to:
Gladys H. Monroy at
(650) 813-5711

201	FULL NAME OF INVENTOR	FAMILY NAME PELLETIER	FIRST GIVEN NAME Jerry	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY Baie D'Urfee	STATE OR FOREIGN COUNTRY Quebec	COUNTRY OF CITIZENSHIP Canada	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 8 Lakeview	CITY Baie D'Urfee	STATE & ZIP CODE/COUNTRY Quebec H9X 3B1, Canada	CAX
202	FULL NAME OF INVENTOR	FAMILY NAME DAS	FIRST GIVEN NAME Manjula	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY Montreal	STATE OR FOREIGN COUNTRY Quebec	COUNTRY OF CITIZENSHIP Canada	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 3484 Stanley, Apt. #205	CITY Montreal	STATE & ZIP CODE/COUNTRY Quebec H3A 1S1, Canada	CAX
203	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
Jerry Pelletier	Manjula Das	
DATE Aug. 21st, 2001	DATE Apr 10, 2002	DATE